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(54) Title: A PACKAGING CELL LINE PRODUCING SIV-PSEUDOTYPED MLV

(57) Abstract

The present invention relates to a packaging cell line packaging an MLV-based recombinant retroviral genome into a particle comprising an SIV envelope, and a pseudotyped retroviral particle produced by the packaging cell line. Both, the packaging cell line and the retroviral particle, when comprising therapeutic genes, are especially useful for somatic gene therapy, preferably in mammals including humans.

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A PACKAGING CELL LINE PRODUCING SIV–PSEUDOTYPED MLV

The present invention relates to retroviral particles carrying the envelopes of heterologous viruses that are discriminating in their binding to host cell membrane bound receptors.

Background of the invention

The use of recombinant retroviruses (RV) for gene therapy has received much attention and currently is the method of choice for the transferral of therapeutic genes in a variety of approved protocols both in the USA and in Europe (Kotani, H., P.B. Newton, S. Zhang, Y.L. Chiang, E. Otto, L. Weaver, R.M. Blaese, W.F. Anderson, and G.J. McGarrity. 1994, Human Gene Therapy 5: 19-28). However, most of these protocols require that the infection of target cells with the RV carrying the therapeutic gene occurs *in vitro*, and successfully infected cells are then returned to the affected individual (Rosenberg, S.A., Anderson, W.F., Blaese, R.M. et al. 1992, Hum. Gene Ther. 3: 75-90; Anderson, W.F. 1992. Human gene therapy. Science 256: 808-813). Such *ex vivo* gene therapy protocols are ideal for correction of medical conditions in which the target cell population can be easily isolated (e.g. lymphocytes). Additionally, the *ex vivo* infection of target cells allows the administration of large quantities of concentrated virus which can be rigorously safety tested before use.

Unfortunately, only a fraction of the possible applications for gene therapy involve target cells that can be easily isolated, cultured and then reintroduced. Additionally, the complex technology and associated high costs of *ex vivo* gene therapy effectively preclude its disseminated use world-wide. Future facile and cost-effective gene therapy will require an *in vivo* approach in which the RV, or cells producing the recombinant virus, are directly administered to the patient in the form of an injection or simple implantation of RV producing cells.

This kind of *in vivo* approach, of course, introduces a variety of new problems. First of all, and above all, safety considerations have to be addressed. Virus will be produced, possibly from an implantation of virus producing cells, and there will be no opportunity to precheck the produced virus. It is important to be aware of the finite risk involved in the use of such systems, as well as trying to produce new systems that minimize this risk.

A further consideration for practical *in vivo* gene therapy, both from safety considerations as well as from an efficiency and from a purely practical point of view, is the targeting of RVs. It is clear that therapeutic genes carried by the viral genome should not be indiscriminately expressed in all tissues and cells, but rather only in the requisite target cell. This is especially important if the genes to be transferred are toxin genes aimed at ablating specific tumour cells. Ablation of other, nontarget cells would obviously be very undesirable.

The ability to target the delivery of genes to predefined cell types is presently difficult, regardless of the method used for gene transfer. The infection spectrum of enveloped viruses is determined by the interaction between viral surface (SU) proteins encoded by the retroviral gene, env, and host cell membrane proteins which act as receptors. Vectors derived from viruses will deliver genes to the same cell types as the original virus does, unless the infection spectrum of the vector virus is modified.

It has long been known that concurrent productive infection of cells with two types of enveloped virus can potentially lead to the production of mixed viral particles or "pseudotypes". These naturally produced "pseudotyped" viral particles may carry the core and genetic information of one virus, and in addition the surface proteins of the other virus (Weiss, R.A. 1993. In: The Retroviridae 2:1-108, ed. J.A. Levy, Plenum Press, New York).

The most commonly used recombinant retroviruses are derived from murine leukaemia virus (MLV); a retrovirus that is able to infect many different cell types. This is due to the expression of the cognate receptor or recognition site, i.e. the cationic amino acid transporter for rodent cells (ecotropic virus) (Kim, J.W., Closs, E.I., Albritton, L.M. and Cunningham, J.M. 1991, Nature 352: 725-728; Wang, H., Kavanaugh, M.P., North, R.A. and Kabat, D. 1991, Nature 352: 729-731) or the phosphate transporter/symporter (Miller, D.G., Miller, D.A.,1994, J.Virol. 68: 8270-8276; van Zeijl, M., Johann, S.V., Classs, E., Cunningham,J., Eddy, R., Shows, T.B. and O'Hara, B. 1994, Proc.Natl.Acad.Sci.USA 91:1168-1172), for this virus on the surface of many different cell types. MLV has been the retrovirus of choice for the production of RVs,

because of the capability of this virus to produce high titre systems, together with the fact that the MLV is a fairly simple virus, and that its biology is well understood. Other retroviruses or enveloped viruses are less promiscuous than MLV in their infection spectrum, but also often give rise to lower titre systems. It is also, at least presently, difficult to construct vectors based upon these virus systems, in part due to the complex nature of their life cycles (Günzburg, W. H. and Salmons, B. 1992, Biochem. J. 283, 625-632).

One way, at least in theory, to combine the ability of viruses to target particular cell types at the level of infection is to create pseudotyped recombinant retroviruses consisting of the core and genome derived from well established MLV based retroviral vectors and the envelope of a second retrovirus or other enveloped virus that shows a limited infection spectrum. Such pseudotyped viruses would have an altered infection spectrum, since they are able to infect the same cells as the second virus providing the envelope and/or surface proteins.

Certain pseudotyped RVs have already been produced in the laboratory by a number of groups using packaging cell lines that produce gag and pol proteins from one virus and env proteins from a second virus.

For example, nontargeted, pseudotyped RVs based upon MLV and carrying the envelope protein of highly promiscous vesicular stomatis virus (VSV) have been described (Yee, J.K., Miyanohara, A., Laporte, P., Bouic, K., Burns, J.C. and Friedmann, T. 1994, Proc. Natl. Acad. Sci. (USA) 91: 9564 - 9568). These vectors give titres higher than 10^9 (cf 10^6 for MLV based RVs) and are more stable, facilitating their concentration. These MLV/VSV pseudotyped RVs show a very wide infection spectrum and are able to infect even fish cells. This suggests, that if such vectors were used for gene therapy they would be capable of infecting many non-target cells, which is very undesirable, especially if the vector is carrying a gene encoding a toxic gene product, for example to treat cancer.

Pseudotyped recombinant viruses based upon MoMuLV (MLV) and carrying the envelope of gibbon ape Leukemia virus (GaLV SEATO-MoMuLV hybrid virion) or the HTLV-I envelope protein (HTLV-I MoMuLV hybrid virion) have been described (Wilson, C., Reitz, M.S., Okayama, H., Eiden, M.V. 19898, Journal of Virology, Vol 63, No. 5, 2374-2378). The GaLV SEATO-MoMuLV hybrid particles were generated at titers approximately equivalent to those obtained with the MoMuLV particles, and the infection spectrum correlates exactly with the previously reported *in vitro* host range of wild type GaLV SEATO, i.e. bat , mink, bovine and human cells. The apparent titers of HTLV-I MoMuLV (1-10 CFU/ml) were substantially lower than the titers achieved with either the MoMuLV or GaLV-MoMuLV recombinant virions. The HTLV-I hybrid virions were able to infect human and mink cells (Wilson, C., Reitz, M.S., Okayama, H., Eiden, M.V. 19898, Journal of Virology, Vol 63, No. 5, 2374-2378).

Object of the invention

It is an object of the present invention to provide pseudotyped retroviral particles which will deliver genes of interest only into target cells, especially into primary blood lymphocytes, without being capable of infecting non-target cells.

Such pseudotyped retroviral particles carrying therapeutic genes will be useful in the treatment of various viral or bacterial infections, as well as cancer.

Summary of the Invention

The invention then, inter alia, comprises the following , alone or in combination:

A packaging cell line packaging a MLV-based recombinant retroviral genome into a particle comprising a SIV envelope;

the packaging cell line as above wherein said envelope protein lacks up to 160 amino acids from the C-terminus;

the packaging cell line as above wherein said envelope protein lacks up to 147 amino acids from the C-terminus;

the packaging cell line as above wherein said retroviral genome comprises heterologous RNA;

the packaging cell line as above wherein the MLV-based recombinant retroviral genome is derived from a MLV-based retroviral vector comprising, in operable linkage,

a) a 5'LTR region originating from MLV comprising the structure U3-R-U5;

b) one or more coding sequences; and

c) a 3'LTR region originating from MLV comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a polylinker sequence carrying at least one unique restriction site and/or, inserted into said polylinker sequence, one or more heterologous DNA fragments, followed by the R and U5 region;

the packaging cell line as above wherein said heterologous RNA and DNA, respectively, is selected from one or more elements of the group consisting of regulatory elements and/or promoters, regulatory elements and/or promoters that are target cell specific in their expression and/or regulatory elements that are regulatable by transacting molecules;

the packaging cell line as above wherein said retroviral genome comprises at least one coding sequence selected from one or more elements of the group comprising marker genes, therapeutic genes, antiviral genes, antitumour genes, cytokine genes;

the packaging cell line as above wherein said coding sequence comprises additionally at least one non coding sequence selected from regulatory elements and/or promoters, regulatory elements and/or promoters that are target cell specific in their expression and/or regulatory elements that are regulatable by transacting molecules, said non coding sequences regulating the expression of at least one of the coding sequences of said retroviral genome;

use of the packaging cell line as above for production of pseudotyped retroviral particles;

a pseudotyed retroviral particle produced by the packaging cell line as above;

the pseudotyped retroviral as above comprising a MLV-based retroviral genome and a SIV envelope;

the pseudotyped retroviral particle as above wherein said SIV wild-type envelope protein lacks up to 160 amino acids from the C-terminus;

the pseudotyped retroviral particle as above wherein said SIV wild type-envelope protein lacks up to 147 amino acids from the C-terminus;

a pharmaceutical composition comprising a pseudotyped retroviral particle as above and/or a packaging cell line as above;

a method for introducing heterologous and/or homologous DNA into cells susceptible to infection by SIV env comprising infecting a cell population *in vivo* and *in vitro* with a retroviral particle as above and/or a pharmaceutical composition as above;

a target cell infected with a retroviral particle as above;

the target cell as above being a primary blood lymphocyte;

the target cell as above being a CD4 expressing cell;

use of the pseudotyped retroviral particle as above and/or the packaging cell line as above for producing a pharmaceutical composition for gene therapy.

Abstract of the Invention

Retroviral vector systems consist of two components:

1) the retroviral vector itself comprises a modified retroviral genome in which the genes encoding for the viral proteins have been replaced by therapeutic genes optionally including marker genes to be transferred to the target cell. Since the replacement of the genes encoding for the viral proteins effectively cripples the virus it must be rescued by the second component in the system which provides the missing viral proteins to the modified retrovirus.

The second component is:

2) a cell line that produces large quantities of the viral proteins, however lacks the ability to produce replication competent virus. This cell line is known as the packaging cell line and consists of a cell line transfected with one or more constructs, e.g. a second or more plasmids, carrying the genes enabling the modified retroviral genome to be packaged. The construct(s) direct(s) the synthesis of the necessary viral proteins required for virion production.

To generate the retroviral genome, the vector is transfected into the packaging cell line. Under these conditions the modified retroviral genome including the inserted therapeutic and optional marker genes is transcribed from the vector and packaged into the modified retroviral particles (recombinant viral particles). A cell infected with such a recombinant viral particle cannot produce new viruses since no viral proteins are present in these cells. However, the recombinat retroviral genome carrying the therapeutic and marker genes is present and these can now be expressed in the infected cell, and in all daughter cells.

The inventors of the present invention have established a packaging cell line packaging a MLV-based recombinant retroviral genome into a particle comprising a SIV envelope.

The present invention thus relates to targeted pseudotyping comprising the use of the envelope from a virus that exhibit a narrow, well defined and specific infection spectrum, namely SIV.

Recently, efficient pseudotyping of a truncated form of human immunodeficiency virus type 1 (HIV-1) envelope with MLV particles has been observed (Mammano, F. et al. 1997, J. Virol. 71: 3341-3345). The incorporation of the HIV-1 envelope into virus particles limits the virus tropism to cells expressing the CD4 marker, thus pointing to a possible *in vivo* utilization of such particles to target therapeutic genes especially to $CD4^+$ cells. Nevertheless, only a truncated form, but not the wild type form of HIV-1 envelope can be efficiently used for pseudotyping since pseudotypes are generated only at very low titers in the presence of a wild-type env construct.

In contrast to this finding it has now been surprisingly found that, however, the wild type envelope of SIV could efficiently pseudotype MLV particles, and yielded titers similar to those obtained with a truncated SIV env construct. SIV is a simian lentivirus closely related to HIV-1 which utilizes the CD4 molecule as a principal receptor on target cells and cause an AIDS-like disease in monkeys. Since, inter alia, primary human blood lymphocytes have been successfully transduced by SIV/MLV pseudotypes and gene transfer has been specifically restricted to the $CD4^+$ subset the inventive pseudoptypes can be used to transduce cells which are susceptible to SIV infection and can be potentially exploited for direct *in vivo* delivery of gene therapy-based therapeutic approaches.

The above mentioned, thus inventive, packaging cell line habours at least one DNA construct coding for proteins required to package the MLV-based recombinant retroviral genome. Accordingly, the core and enzymatic proteins are synthesized from at least one gag and/or pol containing construct and the envelope of SIV from the same or an independent env containing construct or even from the MLV-based recombinant genome.

The wild type SIV envelope may lack up to 160, especially up to 147 amino acids from the C-terminus. Additionally or alternatively, the recombinant retroviral genome comprises preferably heterologous RNA.

The envelope gene of retroviruses encodes two proteins, one of which is a transmembrane protein (TM) which spans the host cell membrane and is involved in the interaction between the envelope and the core of the virus; as well as a second protein which is a surface protein (SU) which is involved in the interaction between the virus particle and the host cell during the initiation of the infection event through its anchor to the TM. It is possible to retain either the MLV TM or the TM domain that interacts with the core of MLV, while replacing the SU, or the SU and the TM domain that interacts with the SU to obtain a packaging cell line which will produce another type of pseudotyped viral particles comprising chimeric envelope proteins.

The invention thus may comprise a packaging cell line as above wherein the sequence encoding the TM domain of said env containing construct has been replaced by a sequence which codes for the TM domain of MLV.

Suitable cell lines may be derived from rodent, human, feline or mink cells, and the gag, pol and env genes may be expressed from a promoter selected from the group of SV40, CMV, RSV, MLV or a house keeping promoter from a cellular gene.

In a further embodiment of the invention, the MLV-based recombinant retroviral genome is derived from a promoter conversion vector:

The retroviral genome consists of an RNA molecule with the structure R-U5-gag-pol-env-U3-R. During the process of reverse transcription, the U5 region is duplicated and placed at the right hand end of the generated DNA molecule, whilst the U3 region is duplicated and placed at the left hand end of the generated DNA molecule. The resulting structure U3-R-U5 is called LTR (Long Terminal Repeat) and is thus identical and repeated at both ends of the DNA structure or provirus. The U3 region at the left hand end of the provirus harbours the promoter. This promoter drives the synthesis of an RNA transcript initiating at the boundary between the left hand U3 and R regions and terminating at the boundary between the right hand R and U5 region. This RNA is packaged into retroviral particles and transported into the target cell to be infected. In the target cell the RNA genome is again reverse transcribed as described above.

According to the ProCon principle a retroviral vector can be constructed in which the righthand U3 region is altered, but the normal lefthand U3 structure is maintained; the vector can be normally transcribed into RNA utilizing the normal retroviral promoter located within the left hand U3 region. However, the generated RNA will only contain the altered righthand U3 structure. In the infected target cell, after reverse transcription, this altered U3 structure will be placed at both ends of the retroviral structure.

If the altered region carries a polylinker instead of the U3 region then any promoter, including those directing tissue specific expression (see below) can be easily inserted. This promoter will then be utilized exclusively in the target cell for expression of linked genes carried by the retroviral vector. Alternatively or additionally DNA segments homologous to one or more celluar sequences can be inserted into the polylinker for the purposes of gene targeting.

In the packaging cell line the expression of the retroviral vector is regulated by the normal unselective retroviral promoter. However, as soon as the vector enters the target cell promoter conversion occurs, and the therapeutic genes are expressed from a tissue specific promoter of choice introduced into the polylinker. Not only can virtually any tissue specific promoter be included in the system, providing for the selective targeting of a wide variety of different cell types, but additionally, following the conversion event, the structure and properties of the retroviral vector no longer resembles that of a virus. This, of course, has extremely important consequences from a safety point of view, since ordinary or state of the art retroviral vectors readily undergo genetic recombination with the packaging vector to produce potentially pathogenic viruses. Promoter conversion (Procon) vectors do not resemble retroviruses because they no longer carry U3 retroviral promoters after conversion thus reducing the possibility of genetic recombination

For a complete disclosure of the ProCon vectors, the content of International application No. PCT/EP95/03445 is completely included within the present application.

In a further embodiment of the invention the packaging cell line thus comprises the MLV-based recombinant retroviral genome which is derived from a MLV-based retroviral vector comprising, in operable linkage,

a) a 5'LTR region originating from MLV comprising the structure U3-R-U5;

b) one or more coding sequences; and

c) a 3'LTR region originating from MLV comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a polylinker sequence carrying at least one unique restriction site and/or, inserted into said polylinker sequence, one or more heterologous DNA fragments, followed by the R and U5 region.

According to the invention the term "polylinker" is used for a short stretch of artificially synthesized DNA which carries a number of unique restriction sites allowing the easy insertion of any promoter or DNA segment. The term "heterologous" is used for any combination of DNA sequences that is not normally found intimately associated in nature.

Gene expression is regulated by promoters. In the absence of promoter function a gene will not be expressed. The normal MLV retroviral promoter is fairly unselective in that it is active in most cell types. However, a number of promoters exist that show activity only in very specific cell types. Such tissue-specific or inducible promoters will be the ideal candidates for the regulation of gene expression in retroviral vectors, limiting expression of the therapeutic genes to specific target cells.

Accordingly, said heterologous DNA fragment as well as the above mentioned heterologous RNA of the retroviral genome is selected from one or more elements of the group consisting of regulatory elements and/or promoters, preferably regulatory elements and/or promoters that are target cell specific in their expression, and/or regulatory elements that are regulatable by transacting molecules.

The target cell specific regulatory elements and promoters are preferably selected from, but not limited to, one or more elements of the group consisting of HIV, Whey Acidic Protein (WAP), Mouse Mammary Tumour Virus (MMTV), ß-lactoglobulin and

casein specific regulatory elements and promoters, which may be used to target human mammary tumours, pancreas specific regulatory elements and promoters including carbonic anhydrase II and ß-glucokinase regulatory elements and promoters, lymphocyte specific regulatory elements and promoters including immunoglobulin and MMTV lymphocytic specific regulatory elements and promoters, and MMTV specific regulatory elements and promoters conferring responsiveness to glucocorticoid hormones or directing expression to the mammary gland, T-cell specific regulatory elements and promoters such as from the T-cell receptor gene and CD4 receptor promoter and B-cell specific regulatory elements and promoters such as immunoglobulin promoter or B29. Said regulatory elements and promoters regulate preferably the expression of at least one of the coding sequences of said retroviral genome.

Another promoter is the HIV promoter or a minimal promoter placed under the regulation of the HIV tat responsive element (TAR) to target HIV infected cells. Targeting will be achieved because the HIV promoter is dependent upon the presence of Tat, an HIV encoded autoregulatory protein (Haseltine, W.A. 1991, Molecular biology of the immunodeficiency virus type 1. FASEB J. 5: 2349-2360.)

Thus, only cells infected with HIV and therefore expressing Tat will be able to produce the peptide encoded by the recombinant genome. Alternatively, the peptide could be expressed from T cell specific promoters such as that from the CD4 or T cell receptor gene. In order to target tumour cells, promoters from genes known to be overexpressed in these cells (for example c-myc, c-fos) may be used.

Coding sequences of the retroviral genome preferably comprises addtionally at least one non coding sequence selected from regulatory elements and/or promoters, more preferably regulatory elements and/or promoters that are target cell specific in their expression, and/or regulatory elements that are regulatable by transacting molecules, said non coding sequences regulating the expression of at least one of the coding sequences of said retroviral genome.

Peptide encoding sequences may be placed also under the transcriptional control of other promoters known in the art. Examples for such promoters are of the group of SV40, cytomegalovirus, Rous sarcoma virus, ß-actin, HIV-LTR, MMTV-LTR, B or T cell specific and tumour specific promoters. The encoded peptide may also be expressed from MMTV promoters such as the ^{MMTV}P2 promoter (Günzburg, W. H., Heinemann, F., Wintersperger, S., Miethke, T., Wagner, H., Erfle, V. and Salmons, B. 1993, Nature 364, 154- 158).

Furthermore, at least one retroviral sequence encoding for a retroviral protein involved in integration of retroviruses may be altered or at least partially deleted.

The MLV-based retroviral vector may contain DNA fragments homologous to one or more cellular sequences.

The retroviral genome especially comprises at least one coding sequence selected from one or more elements of the group comprising marker genes, therapeutic genes, antiviral genes, antitumour genes and/or cytokine genes. Said marker or therapeutic gene may be selected from the group consisting of marker genes which code for proteins such as ß-galactosidase, neomycin, alcohol dehydrogenase, puromycin, hypoxanthine phosphoribosyl transferase (HPRT), hygromycin and secreted alkaline phosphatase, or therapeutic genes which code for proteins such as Herpes Simplex Virus thymidine kinase, cytosine deaminase, guanine phosphoribosyl transferase (gpt), cytochrome P 450, and cell cycle regulatory genes which code for proteins such as SDI or tumor supressor genes which code for proteins such as p53 or antiproliferation genes which code for proteins such as melittin, cecropin or cytokines such as IL-2.

The packaging cell line and the pseudotyped retroviral particle according to the invention may be used for producing a pharmaceutical composition containing a therapeutically effective amount of the packaging cell line and/or of the pseudotyped retroviral particle, together with at least one pharmaceutical carrier or diluent, for somatic gene therapy in mammals including humans. Furthermore, they can be used for targeted integration in homologous cellular sequences.

The retroviral promoter structure is termed LTR. LTRs carry signals that allow them to integrate into the genome of the target cell. Such integrating elements can also contribute to pathogenic changes. Retroviral vectors can carry modified LTRs that no longer carry the signals required for integration. Again this increases the potential safety of these vector systems.

The pseudotyped retroviral particles according to the invention can suitably be administered in the form of an injection or by implantation of the cell line according to the invention, suitably as encapsulated cells producing the pseudotyped retroviral particles according to the invention (see Danish patent application No. 740/95).

The following example describe pseudotyping of MLV-based retroviral genome with SIV envelope, but it will be well understood by a person skilled in the art, that the provided example in no way may be interpreted in a way that limits the applicability of the technology provided by the present invention to this example.

Example

1.1 Plasmids

According to Figure 1A the Mo-MLV Gag-Pol expression construct p*gag-pol*gpt habours the Mo-MLV *gag* and *pol* genes under the control of the Mo-MLV long terminal repeat (LTR) and a SV40 polyadenylation signal (Markowitz, D., Goff, S. and Bank, A. 1988, J. Virol. 62: 1120-1124). The construct lacks ψ packaging sequences as a consequence of a 134-base-pair deletion between the Mo-MLV LTR and *gag* gene. The amphotropic Mo-MLV Env expression construct SV-A-MLV*env* has the A-MLV *env* gene inserted between Mo-MLV LTR sequences and a SV40 polyadenylation signal (Figure 1B; Page, K.A., Landau, N.R. and Littman, D.R. 1990, J. Virol. 64: 5270-5276). The SIV Env expression plasmid pHCMV-SIV*env* (Figure 1B) carries the SIVmac239 *env* gene downstream of human cytomegalovirus early

promoter/enhancer sequences (HCMV). This construct express SIV Env exploiting a Rev-independent pathway of Env expression which relies on a constitutive transport element (CTE) derived from the simian retrovirus 1 (SRV-1) genome (Zolotuhkin, A.S., Valentin, A., Pavlakis, GN. and Felber, B.K. 1994, J. Virol. 68: 7944-7952). This construct has also been used to prepare pHCMV-SIVΔCT*env* (Figure 1B), a variant of pHCMV-SIV*env* with a premature termination codon (TGA) in place of codon 734 of the SIV *env* gene, obtained by site-directed mutagenesis. This results in the truncation of the transmembrane protein from wild-type 354 amino acids (gp41) to 207 (gp28), as already reported by others (Johnston, P.B., Dubay, J.W. and Hunter, E. 1993, J. Virol. 67: 3077-3086).

Finally, the Tat transducing construct pLTSN (Figure 1C) is a derivative of the retroviral vector pLXSN, which contains HIV-1 *tat* under the control of Moloney murine sarcoma virus (Mo-MSV) LTR sequences (Miller, A.D. and Rosman, G.J. 1989, BioTechniques 7: 980-990). The pLXSN EGFP construct (Figure 1C) was used to transduce lymphoid cells ; this construct is a derivative of pLXSN carrying the gene for an enhanced green fluorescent protein (EGFP) driven by the Mo-MSV LTR (Chalfie, M. et al. 1994, Science 263: 802-805). An HCMV-driven β-gal expression plasmid, named pCMVβ-gal, was used in some experiments to check for transfection efficiency.

1.2 Cell culture and transfections

293T human kidney cells were obtained from ATCC, and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco-BRL, Gaithersburg, MD) and 1% L-glutamine. The simian sMAGI cell line (macaque mammary tumor CMMT cells expressing human CD4 and the HIV-1 LTR fused to the β-gal reporter gene), and the human HeLa-CD4-LTR-β-gal cell line (MAGI) were cultured in complete DMEM plus 0.2 mg of G418 (Gibco-BRL) per ml, and 0.1 mg hygromycin per ml (Boehringer Mannheim, Germany) (Chackerian, B.N., Haigwood, N.L. and Overbaugh, J. 1995, Virology 213: 386-394; Kimpton, J. and

Emerman, M. 1992, J. Virol. 66: 2232-2239). Lymphoid cell lines CEMX174 and C8166 were grown in RPMI1640 supplemented with 10% FCS and 1% L-glutamine. Finally, periphal blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Pharmacia-LKB, Uppsala, Sweden) gradient centrifugation as described (Indraccolo, S. et al. 1995, Clin. Immunol. Immunopathol. 77: 253-261), and cultured for 48 h in RPMI1640 supplemented with 10% FCS and 1% L-glutamine, in the presence of phytohemagglutinin (PHA-P, Difco, Detroit, MI), prior to retroviral vector transductions.

The day before transfection, 1.5 x 10^6 cells 293T cells were seeded in 25 cm^2 tissue culture flasks. The cultures were transfected with plasmid DNA using a calcium phosphate precipitation technique (Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989, in: Irvin, N. (ed.) Molecular Cloning: A Laboratory Manual. Cold Spring Habor Laboratory Press: Cold Spring Habor, New York, pp. 16.32-16.36).

1.3 Radioimmunoprecipitation analysis (RIPA)

Forty-eight hours (h) post-transfection, 293T cells were metabolically labeled for 7 h with a mixture of (^{35}S)-methionine and (^{35}S)-cysteine (Promix, Amersham, Little Chalfont, UK; 14,000 µCi/ml, prepared in methionine and cysteine-deficient DMEM), and lysed in RIPA buffer (140 mM NaCl / 8 mM Na_2HPO_4 / 2 mM NaH_2PO_4 / 1% Nonidet P-40 / 0.5% sodium deoxycholate / 0.05% SDS) as described (Mammano, F. et al. 1997, J. Virol. 71: 3341-3345). To control for transfection efficiency among the different samples, pCMVβ-gal was cotransfected, and cell lysate β-gal activity was measured in a β-counter (Packard, Grove Hills, IL). SIV proteins were then immunoprecipitated overnight at 4°C from the cell lysates using a pooled serum from SIV-infected macaques and protein A-Sepharose; bound proteins were released by boiling in 2-mercaptoethanol-containing buffer, and seperated by SDS-PAGE through 10% polyacrylamide gels. To reduce non-specific binding to cellular proteins, the sera were combined with the proteinA-Sepharose, and pre-adsorbed for 2 h with an unlabeled cell lysate from the non transfected cells. Immunoprecipitation of the transfected cell lysates using sera from SIV-uninfected animals did not yield any specific signal.

1.4 Transduction of $CD4^+$ cells

Pseudotyped virions were generated by transfection of 293T cells with 3 μg of the Mo-MLV Gag-Pol expression construct p*gag-pol*gpt, along with 6 μg of either pLTSN or pLXSN EGFP as transducing vector, and 0.1 to 3 μg of the different Env expression constructs. Fresh medium was added to the cultures 12-18 h before the supernatant was collected and passaged through 0.45 μm-pore size filters. To assess the ability of the pseudotyped virions to transduce $CD4^+$ cells, serial dilutions of the filtered supernatants were layered over either CMMT-CD4-LTR-β-gal or HeLa-CD4-LTR-β-gal cells, which had been seeded into 12-well culture plates the day before infection at 3 x 10^4 cells per well. Protamine sulphate (8 μg/ml) (Sigma, St. Louis, MO) was added to the wells, and the cells were kept in a total volume of 1 ml. After 12 h at 37°C, 3 ml of medium was added to dilute the protamine sulphate; 36 h later the cells were washed, fixed and stained as previously described (Kimpton, J. and Emerman, M. 1992, J. Virol. 66: 2232-2239), and blue cells expressing Tat were counted using a light microscope. Titers were expressed as the number of blue foci/ml of added supernatant.

Transduction of CEMX174 and C8166 cell lines was performed by using either cell free supernatants or cocultivation techniques. In the first case, 1 ml of retroviral vector containing supernatant was incubated at 37°C with 2 x 10^5 target cells for 6-9 h in the presence of protamine sulfate (8 μg/ml) with occasional stirring. Lymphoid cells were then pelleted, resuspended in fresh medium and grown for additional 48 h before FACS analysis. For the cocultivation experiments, 10^6 cells in 1 ml RPMI 1640 medium were seeded on transfected 293T cells releasing the different retroviral vectors, and cocultivated for 9 h at 37°C. Suspension cells were then gently removed, transferred to 12-well plates containing 2 ml RPMI 1640 supplemented with 10% FCS and 1% L-glutamine, and cultivated for additional 48 h prior to EGFP detection. In some experiments, transduced CEMX174 cells were cultivated for 2-3 weeks in G418 containing medium (Gibco-BRL, 500 μg/ml active compound), to enrich for vector-transduced cells.

Freshly isolated human lymphocytes were transduced by cocultivation techniques, as detailed above except that, following cocultivation for 9 h, the cells were resuspended in complete RPMI supplemented with 100 U/ml recombinant IL2 (rIL2, EuroCetus, Milan, Italy), and cultured for additional 48 h prior to EGFP detection. In a set of experiments, transduction of PBMC by retroviral vector-containing supernatants was carried out as described for lymphoid cell lines, but resulted in poor efficiency of gene-transfer, compared to cocultivation, and was not further pursued.

1.5 Neutralization assays

Neutralization assays with sera from SIV-infected monkeys were performed following a modification of a previously described protocol (Albert, J. et al. 1993, AIDS Res. Hum. Retroviruses 9: 501-504). As a neutralizing serum a pooled heat-inactivated serum from 3 SIV-infected macaques was used, which contained anti-gp130 Env antibodies, as shown by prior evaluation by Western Blotting. As control serum, a heat-inactivated serum from an uninfected macaque was used. Briefly, 100 µl of diluted serum (six twofold dilutions, starting with the 1:10 dilution) and 100 µl of virus-containing supernatant were mixed. Following 1 h incubation at 37°C, 0.8 ml complete DMEM was added, and the supernatant was applied to sMAGI cells, in the presence of protamine sulfate (8 µg/ml); 36 h after transduction, the number of blue foci was evaluated by β-gal staining.

Neutralization with sCD4 (SmithKline Beecham) was performed as reported (Mammano, F. et al. 1997, J Virol. 71: 3341-3345; Sattentau, Q.J. et al. 1993, J. Virol. 67: 7383-7393). Briefly, 100 µl retroviral vector-containing supernatant was incubated for 1.5 h at 37°C in the presence of 100 µg/ml sCD4. The supernatant was then applied to sMAGI cells. Transduction and titer calculations were carried out as described above.

1.6 Cytofluorographic Analysis

Vector-transduced lymphoid cells were analyzed on an Elite cytofluorometer (Coulter, Hialeah, FL). Forty-eight hours after infection cells were pelleted, washed, and fixed. CEMX174 and C8166 cells were labeled with anti-CD4-PE monoclonal antibody (mAb) (Dako, Glostrup, Denmark). PBMC were labeled with both anti-CD3ECD (Coulter, Hialeah, FL) and anti-CD8PE (Dako) mAbs. Two and three-color immunofluorescence was carried out as reported (Indraccolo et al. 1993, J. Clin Immunol. 13: 381-388), and analyzed by using the PRISM parameter of the Elite cytofluorometer; the negative control setting for each mAb was determined by using labeled Ig of the corresponding isotype.

2.1 Establishment and characterization of SIVmac239 *env* expression plasmids

As detailed above, two SIV env subgenomic constructs have been generated, expressing either wild-type (pHCMV-SIV*env*) or a truncated SIV Env (pHCMV-SIVΔCT*env*) which lacks most of the cytoplasmic domain of the transmembrane protein. To minimize possible instability of truncated Env, this construct was generated by mutating codon 734 of SIV *env* gene, which also naturally undergoes mutations when SIV is passaged in human cell lines (Kodama, T. et al. 1989, J. Virol. 63: 4709-4714).

When 293T cells were transfected with pHCMV-SIV*env*, expression of precursor gp160, as well as gp130 and gp41 cleavage products has been observed. Transfection of pHCMV-SIVΔCT*env* resulted in expression of a smaller precursor protein, which migrated between gp160 and gp130, as well as gp130 and gp28 cleavage products. Gp28 results from the truncation of the cytoplasmic tail of gp41, as already observed by Johnston et al. (Johnston, P.B., Dubay, J.W. and Hunter, E. 1993, J. Virol. 67: 3077-3086). HCMV vector lacking *env* sequences was used as

a negative control in the transfection experiments, and did not yield any specific band in radioimmunoprecipitation studies. Both *env* expression plasmids yielded comparably high levels of Env precursor glycoproteins, which were correctly processed into the expected mature products in transfected 293T cells.

2.2 Generation of infectious virus by pseudotyping of Mo-MLV particles with both wild-type and truncated SIV Env

To determine whether SIV envelope proteins could be functionally incorporated into virions the infectivity of pseudotyped Mo-MLV particles was tested. Pseudotyped viral particles were transiently generated by cotransfection of 293T cells with the constructs shown in Figure 1, and as detailed above. A packageable transcript was provided by cotransfection of pLTSN, which expresses HIV-1 *tat*. As target cells sMAGI cell line, a macaque mammary tumor cell line which expresses human CD4 and habors a β-galactosidase (β-gal) gene under the transcriptional control of HIV-1 LTR sequences, including the Tat responsive element (TAR) (Chackerian, B.N., Haigwood, N.L. and Overbaugh, J. 1995, Virology 213: 386-394) has been used. In this setting, infectious pseudotyped viral particles were expected to transduce the *tat* gene, and thus induce the synthesis of β-gal in the target cells.

The use of both A-MLV Env and of the C-terminally truncated SIV Env, respectively encoded by the SV-A-MLV-*env* and the HCMV-SIVΔ*env* constructs, resulted in the appearance of blue foci following β-gal staining, indicating successful vector transduction. The A-MLV Env, which served as a positive control, yielded titers in the order of 10^4-10^5 transducing units/ml, while the truncated SIV Env generated 10- to 100fold less infectious viral particles. Surprisingly, the wild-type SIV Env, encoded by the HCMV-SIV*env* construct, could also efficiently pseudotype Mo-MLV particles, and yielded titers similar to those obtained with the truncated SIV *env* construct. This result strinkingly differs from what has been observed in case of HIV-1, where efficient gene transduction could be obtained only when the truncated form of the Env complex was used (Mammano, F, et al. 1997, J. Virol. 71: 3341-3345). Positive cells were not detected when no *env* expression construct was used for transfection of 293T cells.

Gene transfer into the human HeLa-CD4 (MAGI) cell line with Mo-MLV/SIV pseudotypes has not been observed. This particular cell line expresses human CD4 and can be infected by T-tropic strains of HIV-1 but not SIV (Kimpton, J. and Emerman, M. 1992, J. Virol. 66: 2232-2239); it is reported to be negative for CCR5 expression and positive for CXCR-4 or another protein that functions as a T-tropic HIV-1 coreceptor (Chackerian, B. et al. 1997, J. Virol. 71: 3932-3939). On the other hand, MAGI cells were efficiently infected by the A-MLV vector, as well as by the Mo-MLV/HIV-1 Env pseudotype (Mammano, F. et al. 1997, J. Virol. 71: 3341-3345). This finding agrees with Marcon et al., who observed that cotransfection of CD4 and CCR5 expression plasmids is necessary to infect HeLa cells with SIVmac239 isolates (Marcon, L. et al. 1997, J. Virol. 71: 2522-2527), and also suggests that coreceptor usage is conserved when the SIVmac239 Env is carried on heterologous particles.

2.3 Mo-MLV/SIV pseudotypes relies on gp130-CD4 interaction transduction of target cells

To verify that the transduction of Mo-MLV particles pseudotyped with SIV Env was mediated by the SIV envelope, target cell infection was inhibited with anti-gp130 antibodies. Pretreatment of Mo-MLV particles pseudotyped by either wild-type or truncated SIV Env with pooled serum from 3 SIV-infected macaques reduced the virus titer measured on sMAGI cells in a dose-dependent manner, whereas a control serum from uninfected macaques did not affect the virus titer of the Mo-MLV/SIV pseudotypes; on the other hand, under identical conditions, the serum from the SIV^+ macaques had no significant effect on the infectivity of particles pseudotyped by A-MLV Env, thus demonstrating that the neutralization was specific for pseudotyping bearing SIV Env.

To further verify that the entry of Mo-MLV particles pseudotyped with wild-type SIV Env was dependent on the interaction between gp130 and CD4, pseudotypes were preincubated with soluble CD4 (sCD4) before the infection of target cells. Pretreatment of Mo-MLV particles pseudotyped by either wild-type or truncated SIV ENV led to an 85% loss of infectivity. Under identical conditions, sCD4 had no significant effect on the infectivity of particles pseudotyped by A-MLV Env.

2.4 Transduction of CD4$^+$ lymphoid cell lines

239T cells were transfected with the *gag-pol* expressor along with the different *env* expression plasmids. As a template for genomic RNA, pLXSN EGFP which carries an enhanced form of the green fluorescent protein (EGFP) driven by the Mo-MLV LTR has been transfected. Green fluorescent proteins are useful markers for gene transfer procedures, because vector-transduced target cells can be easily identified and counted by cytofluorimetrically (Chalfie, M et al. 1994, Science 263: 802-805; Zhang, G., Gurtu, V. and Kain, S.R. 1996, Biochem. Biophys. Res. Commun. 227: 707-711). CEMX174, a CD4$^+$ cell line suitable for infection by different SIV strains, including SIVmac239, has been transduced either by cocultivation or by addition of vector-containing supernatants. It has been found that transduction by cocultivation was generally a more efficient procedure, yielding up to 13.1% transduced CEMX174 cells with the amphotropic vector. Additionally, efficient infection of these cell lines by both SIV pseudotypes took place: the pseudotype with wild-type Env transduced 7.2% target cells, and the one carrying the truncated SIV Env could infect 4.3% CEMX174 cells. Following cocultivation on transfected 293T cells in the absence of any Env, only background values were found. Similar findings were obtained following transduction of the C8166 cell line although the percentage of vector-transduced cells was lower, compared to CEMX174. The pseudotype generated by wild-type SIV Env transduced both human CD4$^+$ cell lines tested slightly better than the one carrying truncated Env.

In 2 experiments, infected CEMX174 cells were grown up to 3 weeks in G418 medium in order to enrich for vector-transduced cells. $EGFP^+$-G418 resistant cultures could be derived from both A-MLV Env and SIV Env pseudotyped Mo-MLV particles, while no culture was obtained from the env-control. These data confirmed the results of the short-term experiments, and demonstrated the stable transduction of target cells by the Mo-MLV/SIV pseudotypes.

2.5 Transduction of human primary lymphocytes

To evaluate whether the SIV/Mo-MLV pseudotype might be useful for targeted transduction of primary $CD4^+T$ lymphocytes PHA-activated peripheral blood mononuclear cells (PBMC) from healthy donors were cocultivated with transiently transfected 293T cells; EGFP was detected on the CD4 and CD8 subsets by FACS analysis.

In 6 independent experiments with different donors, the standard A-MLV vector transduced up to 5% of $CD3^+$ cells (range 1-5%). Both, $CD4^+$ and $CD8^+$ subsets were infected at levels proportional to their percentages of each subset in the transduced population. In a representative experiment, 3.4% $CD3^+$ lymphocytes were transduced by the A-MLV vector; 2.3% belonged to the $CD4^+$ and 1.1% to the $CD8^+$ subsets which accounted, respectively, for 61,8% and 38.2% of the total $CD3^+$ population. Transduction of the cells by SIV wild-type Env pseudotype also resulted in gene transfer into the $CD3^+$ cells with an efficiency of 0.7%. Strikingly, transduced lymphocytes belonged almost exclusively to the $CD4^+$ subset (0.6%). Only background (0.1%) values were found among $CD3^+CD8^+$ cells, similar to those obtained following cocultivation of lymphocytes with the env-control.

In conclusion, these results indicate a restricted tropism of the pseudotyped vector and show that it can be employed for selective gene transfer into human $CD4^+$ lymphocytes.

Claims

1. A packaging cell line packaging a MLV-based recombinant retroviral genome into a particle comprising a SIV envelope.

2. The packaging cell line according to claim 1 wherein said envelope protein lacks up to 160 amino acids from the C-terminus.

3. The packaging cell line according to claim 1 or 2 wherein said envelope protein lacks up to 147 amino acids from the C-terminus.

4. The packaging cell line according to any of the preceding claims wherein said retroviral genome comprises heterologous RNA.

5. The packaging cell line according to any of the preceding claims wherein the MLV-based recombinant retroviral genome is derived from a MLV-based retroviral vector comprising, in operable linkage,

a) a 5'LTR region originating from MLV comprising the structure U3-R-U5;

b) one or more coding sequences; and

c) a 3'LTR region originating from MLV comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a polylinker sequence carrying at least one unique restriction site and/or, inserted into said polylinker sequence, one or more heterologous DNA fragments, followed by the R and U5 region.

6. The packaging cell line according to claim 4 or 5 wherein said heterologous RNA and DNA, respectively, is selected from one or more elements of the group consisting of regulatory elements and/or promoters,

regulatory elements and/or promoters that are target cell specific in their expression and/or regulatory elements that are regulatable by transacting molecules.

7. The packaging cell line according to any of the preceding claims wherein said retroviral genome comprises at least one coding sequence selected from one or more elements of the group comprising marker genes, therapeutic genes, antiviral genes, antitumour genes, cytokine genes.

8. The packaging cell line according to any of the preceding claims wherein said coding sequence comprises additionally at least one non coding sequence selected from regulatory elements and/or promoters, regulatory elements and/or promoters that are target cell specific in their expression and/or regulatory elements that are regulatable by transacting molecules, said non coding sequences regulating the expression of at least one of the coding sequences of said retroviral genome.

9. Use of the packaging cell line according to claims 1-8 for production of pseudotyped retroviral particles.

10. A pseudotyed retroviral particle produced by the packaging cell line according to claims 1-8.

11. The pseudotyped retroviral particle according to claim 10 comprising a MLV-based retroviral genome and a SIV envelope.

12. The pseudotyped retroviral particle according to claim 10 or 11 wherein said SIV wild-type envelope protein lacks up to 160 amino acids from the C-terminus.

13. The pseudotyped retroviral particle according to any of the preceding claims 10-12 wherein said SIV wild-type envelope protein lacks up to 147 amino acids from the C-terminus.

14. Pharmaceutical composition comprising a pseudotyped retroviral particle according to claims 10-13 and/or a packaging cell line according to claims 1-8.

15. A method for introducing heterologous and/or homologous DNA into cells susceptible to infection by SIV env comprising infecting a cell population *in vivo* and *in vitro* with a retroviral particle according to claims 10-13 and/or a pharmaceutical composition according to claim 14.

16. A target cell infected with a retroviral particle according to claims 10-13.

17. The target cell according to claim 16 being a primary blood lymphocyte.

18. The target cell according to claim 16 or 17 being a CD4 expressing cell.

19. Use of the pseudotyped retroviral particle according to claims 10-13 and/or the packaging cell line according to claims 1-8 for producing a pharmaceutical composition for gene therapy.

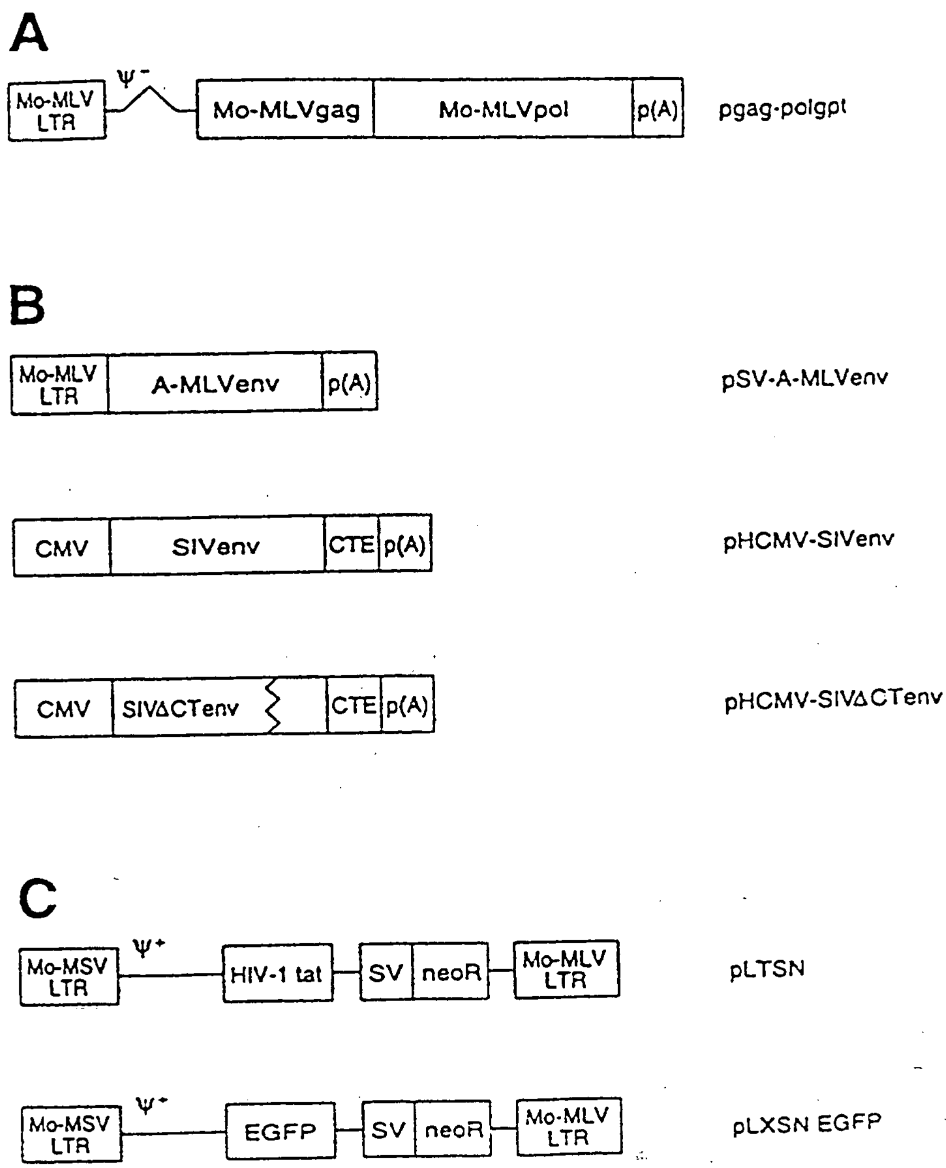
A
Mo-MLV LTR
ψ⁻
Mo-MLVgag
Mo-MLVpol
p(A)
pgag-polgpt
B
Mo-MLV LTR
A-MLVenv
p(A)
pSV-A-MLVenv
CMV
SIVenv
CTE
p(A)
pHCMV-SIVenv
CMV
SIVΔCTenv
CTE
p(A)
pHCMV-SIVΔCTenv
C
Mo-MSV LTR
ψ⁺
HIV-1 tat
SV
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Figure 1

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